Published in final edited form as:

Neuroreport. 2017 December 13; 28(18): 1208–1214. doi:10.1097/WNR.0000000000000895.

Subcellular Localization Patterns of Transglutaminase 2 in Astrocytes and Neurons are Differentially Altered by Hypoxia

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Abstract

The multifunctional protein transglutaminase 2 (TG2) has been widely implicated as a modulator of cellular viability. Specifically, TG2 expression is beneficial to neuronal survival following an ischemic injury, whereas the opposite is true in astrocytes. Furthermore, its role in mediating cell death and survival processes has been suggested to be dependent on its subcellular localization. Therefore, the goal of this study was to examine the subcellular localization patterns of neuronal and astrocytic TG2 in ischemia-relevant conditions. We found that nuclear levels of TG2 were significantly increased in neurons, but reduced in astrocytes, in response to hypoxia. In addition, there were no changes in extracellular TG2 in astrocytes exposed to hypoxia. Thus, these findings demonstrate a difference in the subcellular localization pattern of TG2 in neurons and astrocytes in ischemia-relevant conditions and provide further avenues for investigation into the role of TG2 in mediating cellular viability.

Keywords

Transglutaminase 2; ischemia; localization; nuclear; extracellular; oxygen/glucose deprivation

Introduction

The multifunctional protein transglutaminase 2 (TG2) is a complex contributor to cellular viability [1–4]. TG2 was first identified as a Ca^{2+} -dependent transamidating enzyme, but subsequent studies have demonstrated that it also can function as a GTPase, a protein disulfide isomerase and a scaffold or linker protein [4–6]. Intriguingly the ability of TG2 to protect neurons against ischemic stress is not dependent on transamidating activity [7,8], while the ability of TG2 to facilitate astrocyte migration [9] or potentiate ischemic-induced cell death [10] requires TG2's transamidating activity. Further, its ability to mediate cell death and survival processes has been suggested to be dependent, in part, on its subcellular

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Conflicts of interest

There are no conflicts of interest.

localization [11–15]. In particular, targeting TG2 to the nucleus in various cell lines results in protection against a range of stressors [11–14]. In contrast, tagging TG2 with a nuclear export sequence (NES), which exports TG2 from the nucleus, enhances cell death in response to oxygen/glucose deprivation (OGD) [12]. In neurons, the presence of TG2 enhances cellular viability in response to an ischemic insult [2,7,8], and ischemic injury increases nuclear levels of TG2 [8]. This was true both in mouse brain and in neurons surrounding the infarct area in post-mortem tissue from stroke patients [8].

Extracellular TG2 has also been implicated in cell death and survival processes. In ovarian cancer cells, extracellular TG2 promoted epithelial-to-mesenchymal transition by enhancing their adhesive properties and ability to migrate [16]. Extracellular TG2 can also stabilize extracellular signaling proteins and facilitate adhesion and growth factor-related signaling cascades [17,18]. Astrocytes in post-mortem human brain samples from patients with multiple sclerosis express TG2, which colocalized with the extracellular protein fibronectin [19]. Furthermore, TG2 was externalized by primary rat astrocytes, where it colocalized with fibronectin and promoted adhesion and migration in response to treatment with inflammatory cytokines [20]. Interestingly, contrary to what has been found in astrocytes, there is no indication that TG2 is externalized by neurons.

Although the abovementioned studies demonstrated that the level of nuclear TG2 increases in neurons after injury and that astrocytes can externalize TG2, little else is known about the subcellular localization pattern of TG2 in these cell types [2]. Given the demonstrated importance of nuclear and extracellular localization of TG2 in other cell types, this study examined the subcellular localization pattern of endogenous TG2 in neurons and astrocytes in response to hypoxia, a cellular stressor with relevance in ischemia and other brain injuries [21,22].

Methods and Materials

Animals

Animals were housed and euthanized in accordance with guidelines established by the University of Rochester Committee on Animal Resources. The studies were carried out with approval from the Institutional Animal Care and Use Committee. Timed-pregnant Sprague Dawley rats were obtained from Charles River Laboratories and used for preparation of primary cortical neurons. Colonies of C57Bl/6J mice were maintained in-house and were used for preparation of astrocytes from postnatal day 0 (P0) pups.

Astrocytic and Neuronal Primary Culture

Primary rat cortical neurons were harvested from rat embryos at embryonic day 18 (E18) as previously described [7]. The dissociated cells were plated in poly-D-lysine coated plates with MEM supplemented with 25% fetal bovine serum (FBS), 20 mM glucose, 10 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin. After 5 hrs, the medium was fully replaced with feeding media (Neurobasal media with B-27 supplement and L-glutamine).

Primary cortical astrocytes were harvested at P0 from mice as previously described [2,10]. Astrocytes were maintained in MEM media supplemented with 10% FBS, 6 g/L glucose, 1 mM sodium pyruvate, and 25 µg/mL Gentamicin (Glia MEM) prior to use in experiments.

Neuronal cultures are maintained in Neurobasal media with B-27 supplement without serum which results in very low levels of astrocyte contamination (<5%) based on GFAP staining. The astrocyte cultures are maintained in serum and passaged once before being used. This results in cultures that contain >95% astrocytes [2,10].

Cellular Treatments

For hypoxic treatment of neurons, cells were maintained in feeding media and cultured for 14 hrs in a humidified Coy Laboratory Products Hypoxic Chamber at 37°C with 5% CO₂ and 0.1% O₂. Normoxic control cells were also maintained in feeding media in a humidified incubator at 37°C with 5% CO₂ and ambient levels of O₂.

For hypoxic treatment of astrocytes, cells were cultured in Glia MEM media without serum (SF Glia MEM). Cells were then maintained in a humidified Coy Laboratory Products Hypoxic Chamber at 37°C with 5% CO₂ and 0.2% O₂ for 20 hrs. Astrocytes were kept longer in hypoxia as they are less susceptible to hypoxic injury than neurons [23]. Normoxic control cells were maintained in SF Glia MEM in a humidified incubator at 37°C with 5% CO₂ and ambient levels of O₂.

Neuronal Nuclear Fractionation

After treatments, the neurons were fractionated as previously described, with some modifications [12]. The neurons were washed twice with phosphate buffered saline (PBS) to remove culture medium. Lysis buffer (10 mM pH 7.5 Tris, 10 mM NaCl, 3 mM MgCl₂, 0.05% NP-40, 1 mM EGTA, and protease inhibitors as follows: 1 mM PMSF, 5 µg/mL aprotinin, 5 µg/mL pepstatin, and 5 µg/mL leupeptin) was used to collect the neurons. A portion of this lysate was stored to be used as whole cell sample; the remaining lysate was centrifuged at 400 g for 5 min at 4°C. The supernatant was stored as the cytosolic fraction. The remaining pellet was resuspended in wash buffer (30 mM sucrose, 10 mM pH 6.8 PIPES, 3 mM MgCl₂, 1 mM EGTA, 25 mM NaCl, and protease inhibitors as follows: 1 mM PMSF, 5 µg/mL aprotinin, 5 µg/mL pepstatin, and 5 µg/mL leupeptin) and centrifuged at 400 g for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended and centrifuged again to ensure removal of any remaining cytosolic proteins. The pellet was resuspended in wash buffer and overlaid in 0.5 M sucrose and spun at > 100,000 g for 20 min at room temperature. The supernatant was discarded and the pellet was resuspended in buffer B (300 mM sucrose, 10 mM pH 6.8 PIPES, 3 mM MgCl₂, 1 mM EGTA, 25 mM NaCl, 0.5% Triton X-100, and protease inhibitors as follows: 1 mM PMSF, 5 µg/mL aprotinin, 5 µg/mL pepstatin, and 5 µg/mL leupeptin) and used as the nuclear fraction.

Astrocytic Nuclear Fractionation

After hypoxic/normoxic treatment, astrocytes were trypsinized, pelleted by centrifugation at 3,000 g and resuspended in 1× PBS to wash out the culture medium. Cells were then pelleted again by centrifugation at 3,000 g and resuspended in 500 µL cytosolic extraction

buffer (10 mM HEPES, 1 mM EDTA, 60 mM KCl, 0.075% NP-40, and protease inhibitors as follows: 1 mM PMSF, 5 µg/mL aprotinin, 5 µg/mL pepstatin, and 5 µg/mL leupeptin). The cell lysate was then incubated at 4°C for 30 min, after which a portion of the lysate was reserved to be used as a whole cell sample. The lysate was then spun at 5,000 g for 5 min and the supernatant was collected as the cytosolic fraction. The remaining pellet was then resuspended once more with cytosolic extraction buffer and centrifuged once again at 5,000 g for 5 min. The supernatant was discarded and the remaining pellet was resuspended in 300 µL nuclear extraction buffer (20 mM Tris-HCl pH 7.5, 1.5 mM MgCl₂, 0.2 mM EDTA, 420 mM NaCl, 25% glycerol, 0.5% NP-40, and protease inhibitors as follows: 1 mM PMSF, 5 µg/mL aprotinin, 5 µg/mL pepstatin, and 5 µg/mL leupeptin). The lysate was then incubated at 4°C for 30 min, followed by centrifugation at 12,000 g for 10 min. The remaining pellet was then dissolved in 1× RIPA buffer with phosphatase and protease inhibitors (250 mM Tris-HCl, pH 7.4, 750 mM NaCl, 5% Triton X-100, 5 mM EDTA, 25% glycerol, 0.4% sodium dodecyl sulfate, 20 mM NaF, 2 mM Na₃VO₄, 1 mM PMSF, 5 µg/mL aprotinin, 5 µg/mL pepstatin, and 5 µg/mL leupeptin) and reserved as the nuclear fraction.

For both the neuronal and astrocyte fractions, protein concentrations were determined using the bicinchoninic acid (BCA) assay, and lysate samples were diluted in 1× RIPA buffer with protease and phosphatase inhibitors and 5× reducing stop buffer (60 mM Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, and 0.1% bromophenol blue) at a final concentration of 1 mg/mL.

Surface Biotinylation of Astrocytes and Extracellular Protein Extraction

After hypoxic/normoxic treatment, culture media was removed from astrocytes and cells were rinsed twice with ice cold 1× PBS (pH 8.0). Astrocytes were then incubated on ice with 8 µM EZ-Link Sulfo-NHS-biotin (Thermo Fisher Scientific) for 20 min and then rinsed three times with ice cold 1× PBS. After biotinylation, astrocytes were lysed with 1× RIPA buffer with phosphatase and protease inhibitors (250 mM Tris-HCl, pH 7.4, 750 mM NaCl, 5% Triton X-100, 5 mM EDTA, 25% glycerol, 0.4% sodium dodecyl sulfate, 20 mM NaF, 2 mM Na₃VO₄, 1 mM PMSF, 15 µg/mL aprotinin, 15 µg/mL pepstatin, and 15 µg/mL leupeptin) and the lysate was collected and sonicated. Protein concentration was then determined with a BCA assay, and 100 µg of lysate was incubated with 100 µL Dynabeads M-280 Streptavidin magnetic beads (Thermo Fisher Scientific) that were pre-blocked with ovalbumin (100 mg in 5 mL 1× PBS with 0.5% SDS, and 5 mM EDTA). The lysate and beads were then left to shake at 4°C overnight, and the remainder of the lysate was reserved to measure whole cell protein levels. Biotinylated proteins were then extracted by washing the beads three times with 0.1% SDS in 1× PBS (pH 8.0), incubating in 50 µL 2× reducing stop buffer (2% SDS, 5 mM EGTA, 5 mM EDTA, 25 mM dithiothreitol, 10% glycerol, and 250 mM Tris-Cl, pH 6.8) and then boiling for 15 min. The sample was then collected and reserved for immunoblotting. This sample is defined as the extracellular protein lysate and contains both membrane and ECM bound proteins. Cells that were not incubated with Sulfo-NHS-biotin were subjected to the same protocol as a negative control (not shown).

Immunoblotting

Twenty-five micrograms of protein from the fractionation and biotinylation experiments were incubated at 95°C for 5 min, cooled, and resolved on 10% or 12% polyacrylamide gels, followed by transfer to nitrocellulose membranes. Membranes were blocked in 5% nonfat dry milk in Tris buffered saline with Tween 20 (TBS-T) and subsequently incubated overnight with primary antibodies against TG2 (1:2,500 (astrocytes) and 1:1,000 (neurons); TGM01; rat monoclonal [24]), α -Tubulin (1:5,000; Cell Signaling Technology; rabbit polyclonal), Histone H3 (1:10,000; Thermo Fisher Scientific; rabbit polyclonal), Fibronectin (Sigma; 1:20,000; rabbit polyclonal), or glyceraldehyde phosphate dehydrogenase (GAPDH) (1:1,000; EMD Millipore; mouse monoclonal) in 5% nonfat dry milk in TBS-T. Membranes were then washed with TBS-T and incubated for one hour with HRP-conjugated secondary antibodies (1:2000). After incubation with secondary antibody, membranes were again washed with TBS-T and visualized with enhanced chemiluminescence.

Statistical analysis

A D'Agostino & Pearson test or a Shapiro-Wilk test was conducted to determine whether the data in each experiment followed a normal distribution. A t-test or one-way ANOVA with Tukey's post-hoc for multiple comparisons was used to determine statistical significance for data that followed a normal distribution. A Mann-Whitney test or a Kruskal-Wallis test with Dunn's post-hoc for multiple comparisons was used to determine statistical significance for data that did not follow a normal distribution. All experiments were conducted with at least three biological replicates in triplicate with at least three independent analyses per replicate. A P-value of < 0.05 was considered statistically significant.

Results

Total levels of TG2 are increased in neurons in response to hypoxia, but are not altered in astrocytes exposed to hypoxia

To begin our investigations into the subcellular localization of TG2, it was first necessary to investigate whether or not hypoxic treatment altered total cellular levels of TG2. TG2 contains several putative hypoxic response elements (HREs) in its promoter region, indicating that its expression may be induced by hypoxic signaling mechanisms [25]. Furthermore, previous animal and cellular studies have shown that ischemic insults increase TG2 protein and mRNA levels [7,8,26,27]. In order to recapitulate these findings, neurons were exposed to 14 hrs of hypoxia and collected for immunoblotting. There was a significant increase in TG2 levels in neurons exposed to hypoxia compared to those maintained in normoxia (Figure 1A, B). Next, astrocytes were exposed to 20 hrs of serum starvation/normoxia or serum starvation/hypoxia, after which protein was collected for immunoblotting. In this paradigm, there was no difference in the amount of TG2 between astrocytes exposed to normoxia and hypoxia (Figure 1C, D). These results show that after hypoxic treatment TG2 expression is increased in neurons while it remains unchanged in astrocytes.

Nuclear levels of TG2 are increased in neurons but reduced in astrocytes in response to hypoxia

To determine the localization of TG2, neurons were exposed to 14 hrs of hypoxia and fractionated. In response to hypoxia, the nuclear levels of TG2 were significantly increased compared to normoxic controls (Figure 2A, B). Although total levels of astrocytic TG2 were not affected by exposure to hypoxia (Figure 1C, D), the distribution of TG2 within astrocytes could still be altered by hypoxic treatment. Thus, a nuclear fractionation was also performed with astrocytes exposed to either serum starvation/normoxia conditions or serum starvation/hypoxia for 20 hrs. It should be noted that due to the morphological and functional differences of neurons and astrocytes the hypoxia and fractionation paradigms could not be identical. Despite these differences, however, the relative changes in nuclear TG2 levels in astrocytes and neurons in response to hypoxia are still able to be compared. In both fractionation experiments, the non-nuclear and nuclear fractions were confirmed by blotting for the appropriate loading control proteins (GAPDH or tubulin for non-nuclear fractions and histones for nuclear fractions), which were also used to normalize TG2 expression levels. In astrocytes hypoxia significantly reduced nuclear TG2 protein levels by approximately 75% compared to nuclear levels in normoxia (Figure 3A, C). These results show that hypoxic treatment of neurons and astrocytes results in differential subcellular localization of TG2. In neurons, TG2 nuclear levels increase with hypoxic treatment while in astrocytes nuclear TG2 protein levels are reduced.

Extracellular levels of TG2 are not altered in astrocytes in response to hypoxia

We next examined how hypoxia affects external levels of TG2 in neurons and astrocytes. Neuronal membrane bound TG2 was not detected by immunocytochemistry (data not shown) and thus further analyses were not carried out. Astrocytes were subjected to either serum starvation/normoxia or serum starvation/hypoxia, followed by surface biotinylation to isolate extracellular proteins. In agreement with previous studies using rat astrocytes [20], TG2 was present extracellularly in primary mouse astrocytes. The protein level of extracellular expression of TG2, however, was not altered in response to hypoxia (Figure 3B, C). This result shows that TG2 is expressed extracellularly in astrocytes and this expression is not altered by hypoxia.

Discussion

The functions and characteristics of TG2 that contribute to cellular viability are an important avenue of investigation. TG2 subcellular localization in particular can significantly impact its ability to mediate intracellular signaling processes that contribute to cell death and survival [11,12,14,15]. In the present study we have shown that hypoxia, a central nervous system (CNS) injury-relevant stressor, increases neuronal expression of TG2, but has no effect on the expression of TG2 in astrocytes. An overall increase in TG2 expression has been previously reported in animal models of ischemic injury. TG2 mRNA and protein levels were increased in the hippocampus and blood vessels of gerbils that underwent a global cerebral ischemia injury [26,28]. Additionally, rats and mice that underwent a middle cerebral artery occlusion showed increased TG2 mRNA and protein levels in the cortex and hippocampus [27,29]. To better understand the functions of TG2 after an ischemic injury,

studies focused separately on neuronal or astrocytic TG2. In neurons treated with OGD, TG2 levels increased, and this increased expression was protective [7]. The neural protection by TG2 was further investigated in animal models. Stroke volumes following middle cerebral artery ligation (MCAL) were significantly reduced in mice overexpressing human TG2 selectively in neurons compared to wild type littermates [8]. To extend these findings, MCALs were performed in TG2 knock out mice (TG2^{-/-}). Similarly and surprisingly, infarct volumes were smaller compared to wild type mice [2]. To understand this finding, neurons and astrocytes from TG2^{-/-} mice were cultured separately and subjected to OGD. Neurons from TG2^{-/-} mice showed increased cell death in response to OGD compared to wild type neurons. However, the opposite was seen in cultured astrocytes from TG2^{-/-} mice treated with OGD [2]. Given these findings, it can be suggested that the increase in TG2 expression in neurons following OGD [7] or hypoxia (this study) may be a protective response. The expression of astrocytic TG2 after hypoxia remains unchanged perhaps because TG2 does not have a protective role in this cell type.

In this study, we also found that nuclear levels of TG2 are increased in the neurons but reduced in astrocytes in response to hypoxia. Previous studies in other cell types have shown that nuclear expression of TG2 is protective. In a study using HEK293 cells, TG2 tagged with an NLS showed an increase in cell viability after OGD treatment [13]. In a mouse model, neuronal TG2 nuclear localization was increased after a MCAL [8]. The accumulation of TG2 in the nucleus was time dependent after the MCAL, suggesting that TG2 accumulates as the injury progresses. This neuronal nuclear accumulation of TG2 is also present in human stroke cases [8].

Finally, the extracellular localization of TG2 in astrocytes was also investigated. Hypoxic treatment does not alter extracellular TG2 expression. Previous work has found that TG2 can be expressed at the surface of cells [30]. Extracellular TG2 can bind to various extracellular matrix proteins [31], increase focal adhesion [30,32], and mediate integrin signaling [33,34]. In our studies, extracellular TG2 did not change. This localization of TG2, therefore, might not be contributing to astrocytic viability after OGD.

These studies demonstrate for the first time that nuclear levels of TG2 are increased in neurons, but reduced in astrocytes, in response to hypoxia. The specific mechanisms by which TG2 contributes to cellular viability processes within neurons and astrocytes are still under investigation. Furthermore, we do not know the mechanism by which hypoxia modulates nuclear levels of TG2. Future studies are needed to delineate the specific signaling pathways that are modulated by TG2 in the different cellular compartments and their effects on cell viability. Overall, these studies have provided us with new information about TG2 in neurons and astrocytes, and have opened several key avenues for further exploration. A greater understanding of these mechanisms will better inform the specific role of TG2 in different cell types under stressed conditions.

Acknowledgments

The authors would like to thank Dr. Robert Freeman for the use of his Coy hypoxic chamber and Dr. Elaine Smolock for her comments in the preparation of this manuscript. This work was supported by the National

Institutes of Health (Grant Numbers R01 NS065825 [G.V.W.J.], F31 NS081894 [J.F.] and F31 NS084572 [L.Y.M.]).

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Figure 1. Total cellular TG2 levels increase in neurons, but not in astrocytes, in response to hypoxia

(A) Representative immunoblot of TG2 expression in neurons exposed to 14 hrs of hypoxia (hyp) or normoxia (norm). (B) Quantification of (A). TG2 protein expression was normalized to GAPDH as a loading control. TG2 expression was normalized to normoxic controls ($n = 6$). (C) Representative immunoblot of TG2 expression in astrocytes exposed to 20 hrs of serum starvation/normoxia (norm) or 20 hrs serum starvation/hypoxia (hyp). (D) Quantification of (C). TG2 protein expression was normalized to tubulin as a loading control. TG2 expression in hypoxia was normalized to expression under normoxic conditions ($n = 15$). Lines in between lanes represent the removal of intervening lanes on the same blot. Results are presented as mean $\pm$ SEM. (** $p < 0.005$; n.s. – not significant).

Figure 2. Neuronal nuclear levels of TG2 increase in response to hypoxia

(A) Representative immunoblots of non-nuclear/cytosolic and nuclear fractions from neurons exposed to 14 hrs of hypoxia or normoxia. (B) Quantification of the immunoblots; non-nuclear levels of TG2 are normalized to GAPDH and nuclear levels are normalized to histone. Results are presented as mean $\pm$ SEM. (* $p < 0.05$, $n = 4$)

Figure 3. Astrocytic nuclear levels of TG2 are reduced in response to hypoxia

(A) Representative immunoblot of non-nuclear/cytosolic and nuclear fractions from astrocytes exposed to 20 hrs of serum starvation/normoxic conditions (norm) or 20 hrs of serum starvation/hypoxic conditions (hyp). (B) Representative immunoblot of whole cell lysate and extracellular proteins extracted by surface biotinylation of astrocytes exposed to the same conditions as (A). (C) Quantification of (A) and (B). Non-nuclear TG2 levels were normalized to tubulin, nuclear TG2 levels were normalized to H3 histones, and extracellular TG2 levels were normalized to tubulin in the corresponding whole cell lysate. Data are presented as TG2 expression in hypoxia relative to TG2 expression in normoxia (dotted line). Results are presented as mean $\pm$ SEM. (****p < 0.0001, n = 4).